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(54) Human erythropoietin gene: high level expression in stably transfected mammalian cells.

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PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE USA, vol. 83, no. 17, September 1986, pages 6465-6469, Washington, DC, US; J.S. POWELL et al.: "Human erythropoietin gene: High level expression in stably transfected mammalian cells and chromosome localization"

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Description

Field of the Invention

This invention relates generally to the field of genetic engineering, particularly to the expression of glycoprotein products of recombinant genes, and more particularly to the expression of high levels of biologically active human erythropoietin from stably transfected cells.

Background of the Invention

The hormone erythropoietin plays a major role in regulating erythropoiesis, the formation of red blood cells, and deficiencies of erythropoietin result in anemia. Detailed studies of the hormone and attempts at replacement therapy have been difficult due to the scarcity of purified material.

Normal production of human red blood cells requires the secretion of erythropoietin by the kidney, apparently as the mature glycoprotein. In the steady state this hormone circulates in the blood at a concentration of 10 to 18 milliunits (128-230 picograms) per milliliter, and with the stimulus of severe tissue hypoxia (oxygen deficiency) the levels may increase as much as 1000-fold. The elevated hormone level triggers proliferation and differentiation of a population of receptive stem cells in the bone marrow, stimulates hemoglobin synthesis in maturing erythroid cells, and accelerates release of red cells from the marrow into circulation, thereby increasing the red cell mass and ameliorating the hypoxic conditions. Patients with deficiencies of erythropoietin, such as those with chronic renal failure, often suffer severe anemia.

Erythropoietin is a glycoprotein of 34-38 kd with approximately 40% of its molecular weight provided by carbohydrate. At least one disulfide bridge is required for activity. Little is known about the structure of this hormone, and the details of its synthesis are not well understood. Recent isolations of cDNA and genomic clones provide opportunities to analyze control of erythropoietin production, but the expression of biologically active human erythropoietin in sufficient quantities for replacement therapy has not been achieved.

Summary of the Invention

Pursuant to this disclosure, biologically active human erythropoietin can be expressed at high levels (at nominal titers exceeding two million Units per liter of supernatant) from stably transfected mammalian cell lines. Thus, an abundant source of purified human erythropoietin for clinical applications is provided. Surprisingly high expression of erythropoietin is achieved by transfecting host cell lines with the 2.4 kb Apa I restriction fragment of the human erythropoietin gene. The sense strand of the Apa I restriction fragment has a nucleotide sequence corresponding to that shown in FIGURE 1.

Brief Description of the Drawings

FIGURE 1 is a schematic representation of the subject 2426 bp Apa I restriction fragment that contains the human erythropoietin gene sequences;

FIGURE 2 depicts a representative plasmid expression vector (pD11-Ep) that contains the 2426 bp Apa I restriction fragment; and,

FIGURE 3 depicts another expression vector (pBD-EP) carrying the subject Apa I restriction fragment.

Detailed Description of the Preferred Embodiment

In the preferred embodiment, a genetically engineered construction of the Apa I restriction fragment shown in FIGURE 1 is inserted into a mammalian expression vector, such as those shown in FIGURES 2 and 3, and introduced into mammalian cell lines to develop stably transfected cells that produce large amounts of biologically active human erythropoietin. The Apa I restriction fragment of the human erythropoietin gene was selected to maximize efficient transcription of erythropoietin messenger RNA and effective translation and post-translational modification of the RNA into mature biologically active erythropoietin glycoprotein. Specifically, at the 5' end of the erythropoietin gene it was important to remove interfering sequences but retain enhancing sequences. Introns were retained in the Apa I restriction fragment in order to include potentially significant enhancing sequences. At the 3' end of the gene some 3' untranslated

sequences were retained to optimize putative regulating sequences. The enhanced expression provided by the Apa I fragment is demonstrated by the consistently high levels of erythropoietin expression described in the following working examples, using this fragment in concert with two promoters and two cell lines.

The following Examples are presented to illustrate the advantages of the present invention and to assist one of ordinary skill in making and using the same. These Examples are not intended in any way to otherwise limit the scope of the disclosure or the protection granted by Letters Patent hereon.

EXAMPLE 1

Isolation of genomic clones

A human genomic library in bacteriophage lambda (Cell 15:1157-1174, 1978) was screened using low stringency hybridization conditions and mixtures of oligonucleotide probes as described in Cell 38:287-297, 1984, hereby incorporated by reference.

Oligonucleotide mixtures were prepared using an Applied Biosystems synthesizer and end-labeled using ^{32}p-ATP and T4 polynucleotide kinase. The synthetic oligonucleotides were designed to correspond to portions of the amino terminal amino acid sequence of:

H_2N-Ala-Pro-?-Arg-Leu-Ile-Leu-Asp-Ser-Arg-Val-Leu-Glu-Arg-
Tyr-Leu-Leu-Glu-Ala-Lys-Glu-Ala-Glu-?-Ile-Thr-Asp-Gly-Gly-Ala
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obtained by Yanagawa et al. (J.Biol.Chem. 259:2707-2710, 1984) for the human protein purified from urine of patients with aplastic anemia. To reduce the degeneracy of the codons for the amino acid sequence of this region, the codon usage rules of Grantham et al. (Nucleic Acids Research 8:43-59, 1981) and Jaye et al. (Nucleic Acids Research 11:2325-2335, 1983) were employed. These rules take into account the relatively rare occurrence of CpG dinucleotides in DNA of vertebrates and avoid, where appropriate, potential A:G mismatch pairings. At amino acid position 24, an asparagine was placed as most likely (J.Biol.Chem. 259:2707-2710, 1984). For the amino acids Glu-Ala-Lys-Glu-Ala-Glu-Asn, 2 pools of 72 sequences each were synthesized to correspond to the predicted codons. Thus, one pool was TT(c/t)TC-(a/g/t)GC(c/t)TC(c/t)TT(a/g/t)-GCTTC for the 20 nucleotide probe, and the second pool replaced a T with a C at position 18. For the amino acids Glu-Asn-Ile-Thr-Asp-Gly, one pool of sequences (AGC TCC TCC ATC AGT ATT ATT T[c/t]) was constructed for the 23 nucleotide probe.

Plaques which hybridized to the oligonucleotide probes were rescreened at lower density until pure. After initially positive phage clones were plaque-purified, additional sequence information for the erythropoietin gene was published by Jacobs et al. (Nature 313:806-310, 1985). Oligonucleotides were constructed from this information and used to confirm the identity of the positive clones. After Eco RI restriction enzyme digestion of the positive clones, insert DNA was gel-purified and ligated by standard techniques into pUC 13 plasmid previously restriction-digested with Eco RI. DNA sequence was determined by dideoxynucleotide chain termination (Proc.Natl.Acad.Sci.USA 74:5463-5467, 1977) using dATP (α-^{35}S) and the 17-mer universal primer or, in selected regions, specific oligonucleotide primers. ^{32}p-ATP was from ICN; enzymes were from New England Biolabs or Bethesda Research Laboratories.

Approximately 4.8 X 10^6 bacteriophage were screened by hybridization of replicate nitrocellulose filters. Three different clones remained positive through plaque purification, and the DNA insert was characterized by restriction mapping and partial dideoxynucleotide sequencing. Two of the three clones contained apparently complete information for the erythropoietin gene. The restriction map and sequence for the 2426 bp Apa I fragment of these clones is shown in FIGURE 1 and was essentially the same as that recently published by Jacobs et al. (Nature 313:806-810, 1985) for the gene for human erythropoietin. The low frequency of phage isolates containing the erythropoietin gene in this amplified library, one in approximately 2x10^6 bacteriophage, is consistent with the suggestion that erythropoietin exists as a single copy in the human genome. Southern blot hybridization of total human genomic DNA with the Apa I fragment or other restriction fragments of the erythropoietin gene indicated only a single hybridizing band with no additional regions of highly homologous DNA.

EXAMPLE 2

Selection of the Apa I restriction fragment.

For construction of expression vectors the Apa I restriction fragment of the erythropoietin gene was obtained using techniques described in Proc. Natl.Acad.Sci.USA 74:5463-5467, 1967, hereby incorporated by reference. Briefly, isolated phage DNA was digested with the restriction enzyme Apa I (Bethesda Research Laboratories) followed by separation on a 1% agarose gel and isolation by electroelution, phenol extraction and ethanol precipitation. The fragment was confirmed by partial sequencing as in Example 1.

Referring to FIGURE 1, the inserted Apa I restriction fragment contained 58 bp of 5' untranslated sequences (nucleotides 0001-0058) followed by sequences coding for a putative 27 amino acid signal peptide, the mature protein, four putative intervening sequences, and 222 bp of 3' noncoding DNA sequence. At the 5' end of the gene the Apa I site is located 58 base pairs upstream from the ATG start codon (0058) for the protein sequence. This 5' site (0001) was selected to avoid a false start site just upstream from the Apa I restriction site while including putative regulatory sequences considered to be important for efficient ribosomal binding and processing. The Apa I site at the 3' end (2426) was selected to include putative processing signals in most of the 3' untranslated sequences while removing further downstream sequences. The complete erythropoietin gene, including intron sequences, was used in order to include potential regulatory or enhancing sequences located in introns that might contribute to erythropoietin gene expression or protein modification and secretion.

EXAMPLE 3

Construction of expression plasmids carrying the Apa I restriction fragment.

The 2426 bp Apa I restriction fragment containing the intact human erythropoietin gene was inserted into two expression vectors, the pD-11 and pBD constructs, each of which is based on a different mammalian promoter.

The plasmid expression vector pD11 was derived from a previously described plasmid (Nucleic Acids Research 13:841-857, 1985, expressly incorporated herein by reference) and contained the simian virus 40 (SV-40) enhancer sequences and origin of replication as well as the adenovirus-2 major late promoter and tripartite leader sequences. The Apa I fragment of human erythropoietin genomic sequences (FIGURE 1) was gel-purified, and single stranded ends were filled in by treatment with T4 DNA polymerase. Bam HI linkers were ligated to both blunt ends, and the construct was inserted into a unique B-Bam HI restriction site of pD11 in order to direct transcription of the erythropoietin gene from a strong promoter.

The structure of the resulting expression plasmid pD11-Ep carrying the Apa I fragment (Ep) is depicted in FIGURE 2. The plasmid pD11 contains 350 bp of the adenovirus left-terminus (0-1), the origin and enhancer sequences from SV-40 (E), the adenovirus major late promoter (MLP), the adenovirus-2 tripartite leader (L1-3) and third leader 5' splice site (5' ss), an immunoglobulin 3' splice site (3' ss), and the late SV-40 polyadenylation signal (pA) in the Eco RI (RI) restriction site of pML. Recombinant plasmids were cloned in E. coli HB101 and purified by isopycnic centrifugation in cesium chloride. The expression plasmid pD11-Ep is approximately 6500 bp in length. The construction was confirmed by restriction mapping and partial dideoxynucleotide sequencing.

pBD contains the MT-I promoter sequence (Glanville, Durham & Palmiter, Nature 292:267-269, 1981, expressly incorporated herein by reference) and a DHFR selectable marker sequence carried in pUC plasmid. Referring to FIGURE 3, the 2426 bp Apa I restriction fragment (EP) was inserted into a unique Sma I restriction site in pBD to make expression plasmid pBD-EP. The DHFR sequence in pBD-EP was associated with origin and enhancer sequences from SV40 and with Hepatitis B Surface Antigen polyadenylation sequences. pBD-EP was processed essentially as described above for pD11-EP.

While plasmid vectors were used in these confirming experiments, it is contemplated that viral or retroviral expression vectors can likewise be used to introduce the Apa I erythropoietin gene fragment into host cell lines. Suitable DNA viruses for this purpose would include adenovirus or BPV (Bovine Papilloma Virus). Suitable retroviral vectors are also known and available. It is understood that such a retroviral (RNA) vector would carry the anti-sense strand of the Apa I restriction fragment, that is, one having a sequence corresponding to the RNA transcribed from the sense strand shown in FIGURE 1 or to an allele thereof. The retroviral vector would also include sequences for trans-acting factors required for reverse transcription of the viral genome and integration of the DNA form of the virus into the host genome.

EXAMPLE 4

Transfection of mammalian cells.

Each of two mammalian cell lines were transfected with each of th pD11-EP and pBD-EP constructs. Mammalian cell lines, COS-7 (monkey kidney) and BHK (baby hamster kidney), were maintained in Dulbecco's modified essential medium containing 10% fetal calf serum. Cells were passaged and when 50-70% confluent were transfected by the calcium phosphate method (Virology 52:456-467, 1973). BHK derives from kidney epithelial cells, generally regarded as the most likely cell that produces erythropoietin in the natural state when a mammal is anemic or hypoxic. Thus, these cells might recognize critical regulatory sequences in the Apa I fragment of the erythropoietin gene, and then effectively process and produce the erythropoietin glycoprotein.

For transient expression of cells in a 100 mm culture dish a total of 20 µg DNA was used: 10 µg of pD11-EP plasmid containing the erythropoietin gene and 10µg of carrier salmon sperm DNA. After 48 hours the supernatant was collected, centrifuged at 400 g for 10 minutes to remove cells and debris, and frozen at -20°C. The cells were harvested separately. The results of transfections with pD11-EP alone for transient expression are shown in Table 1. Data are from 3 experiments for each cell type.

TABLE 1

Mammalian cells	Erythropoietin per ml of culture	
	Micrograms protein	Units (in vitro bioassay)
BHK	3.4±0.2	270±16
COS-7	3.2±0.4	255±32

The observed levels of erythropoietin secreted into the supernatant of either the COS-7 or BHK mammalian cell lines were approximately 80 times higher than those previously reported for transient expression of a cDNA coding for erythropoietin or for transient expression of other constructs using intact erythropoietin genes.

To establish stable cell lines producing high levels of erythropoietin, either COS-7 or BHK cells were cotransfected with the pD11-Ep plasmid and pDHFR-1a, a plasmid containing a cDNA for dihydrofolate reductase in a similar mammalian expression vector. The transfection procedure was modified so that 5 µg of pD11-Ep plasmid, 5 µg of pDHFR-1a plasmid, and 10 µg of carrier DNA were cotransfected. After additional incubation for 18-24 hours, varying concentrations of methotrexate (10 nM to 1 mM) were added to the cultures. Cells that incorporated the DHFR gene would be viable in the selective medium. After incubation for several more days, viable colonies resistant to methotrexate were isolated, passaged and screened for the presence of erythropoietin bioactivity in the supernatant. Approximately half of the methotrexate-resistant colonies that were assayed secreted detectable erythropoietin activity.

To establish stable cell lines with the expression vector pBD-EP, BHK cells were transfected by the calcium phosphate method. After 18-24 hours, these cultures wore subjected to concentrations of methotrexate varying from 1 µ M up to 1 mM. After incubation for several more days, viable colonies resistant to these relatively high levels of methotrexate were isolated, passaged and screened. In this series, all of the methotrexate-resistant colonies that were assayed secreted detectable erythropoietin activity.

In order to optimize the expression of the transcriptional unit containing the erythropoietin gene and the DHFR gene, BHK cell lines containing either pBD-Ep or pD11-Ep and secreting high levels of erythropoietin were passaged several times into increasing concentrations of methotrexate (Nature 316:271-273, 1985). However, rather than a gradual increase in the selective pressure on the cell lines by small incremental steps in the concentration of methotrexate, the cells were immediately challenged with very high levels of methotrexate (i.e., 1 mM). Only a few cells survived, but those cells would have incorporated the plasmid construction into a region of DNA particularly advantageous for expression (a so-called "hot spot") and/or would have many copies of the constructed transciptional unit. Thus, the highest producing cell lines were selected in one step. Cell lines (including F 7.2 and S 5.2 listed in Table 2) were considered stable if erythropoietin production remained high for more than 15 passages in the absence of methotrexate selective pressure.

Amounts of erythropoietin activity in the cell pellets could not be determined due to the presence of significant inhibitors of the assay in the cell extracts. Consequently, the results shown in Table 2 do not analyze the intracellular levels of erythropoietin protein but rather the amount of erythropoietin protein produced and secreted into the supernatant by the cell lines.

TABLE 2

Expression of Recombinant Erythropoietin From Stably Transfected BHK Cell Lines

	Erythropoietin per ml of supernatant	
Cell Line	Micrograms protein	Units (in vitro bioassay)
pBD-EP		
F 1.1	12.4	970
F 3.4	32.0	2500
F 6.1	79.6	6210
F 7.2	84.1	6728
pD11-EP		
S 1.2	6.4	500
S 2.4	64.2	5000
S 5.2	82.1	6400

The observed amounts of erythropoietin secretion correspond to nominal rates of up to almost seven million Units per liter. Such nominal rates are determined by multiplying the observed yield per ml by one thousand to give an anticipated scaled-up production yield per liter.

The observed amounts of erythropoietin secretion using the Apa I fragment were on the order of 300 times greater than those previously reported (Lin et al., Proc.Natl.Acad.Sci.USA 82:7580-7584, Nov. 1985) for a stably transformed CHO cell line containing a different genomic fragment of the human erythropoietin gene.

Control experiments for these transfection assays included supernatants from nontransfected cells and parallel cultures of cells transfected with plasmids containing DNA encoding other proteins, including bacterial chloramphenicol acetyl transferase and human coagulation protein, factor IX. None of the control cultures, mock transfections, or cultured cells transfected with other genes had detectable erythropoietin activity. It was also noted in the above series of experiments with the erythropoietin gene that the levels of expression obtained for selected cell lines were not related to whether the selectable marker was cotransfected along with the erythropoietin gene or was inserted into the Apa I fragment-containing plasmid prior to transfection (data not shown).

Other representative transfection methods suitable for practicing this invention include DEAE-dextran mediated transfection techniques, lysozyme fusion or erythrocyte fusion, scraping, direct uptake, osmotic or sucrose shock, direct microinjection, indirect microinjection such as via erythrocyte-mediated techniques, and/or by subjecting the host cells to electric currents. By transfection is meant the transfer of genetic information, and specifically the information encoded by the Apa I restriction fragment of a human erythropoietin gene, to a cell using isolated DNA, RNA, or synthetic nucleotide polymer. The above list of transfection techniques is not considered to be exhaustive, as other procedures for introducing genetic information into cells will no doubt be developed. The Apa I restriction fragment will typically be operably linked (ligated) to other nucleic acid sequences such as promoter, enhancer, and polyadenylation sequences, prior to transfection. While host cell lines of mammalian origin are described, and kidney epithelial cells are considered to be particularly preferred, it is contemplated that other eukaryotic as well as prokaryotic (bacteria or yeast) host cell lines can be employed in the practice of this invention; very recent introductions of mammalian genes into plant cells offer the potential for employing plant or algal cells as well.

EXAMPLE 5

Erythropoietin expression from transfected cell lines.

The recombinant erythropoietin protein secreted into the supernatant of the transfected cell lines was biologically active, and large amounts of the hormone were secreted: up to 7000 units per milliliter.

The in vitro assay for erythropoietin biological activity was based on the formation of erythroid colonies (from CFU-E; erythroid colony-forming cells) in cultures of mouse bone marrow cells in plasma clot (Blood Cells 4:89-103, 1978). The sensitivity of this assay is about 5 milliunits/ml. The erythropoietin used as the standard for assay was a partially purified preparation from plasma from anemic sheep (Connaught, Step 3 Ep, Lot 3026). Supernatants were assayed from passaged cell lines grown for 24 hours in fresh medium without methotrexate. The supernatant was diluted 1:200 with medium, and amounts beween 1 and 10 microliters were added per milliliter of assay culture containing 2 x 10^5 marrow cells, 10% bovine citrated plasma, 20% fetal calf serum, 1% bovine serum albumin, and 1.6% beef embryo extract (Gibco). After incubation for 36 to 48 hours the plasma clots were fixed on microscope slides, stained with benzidine for hemoglobin, and erythroid colonies were enumerated. In the absence of added erythropoietin, no CFU-E-derived colonies were detected. Optimal erythroid colony growth (100-150 CFU-E detected per 2 x 10^4 marrow cells) was observed routinely with 50 mU (0.64 nanograms) erythropoietin per ml of culture. Large amounts of erythropoietin hormones were secreted into the supernatant of the transfected cell lines, see Tables 1 and 2, up to 7000 units per milliliter. Assuming the recombinant erythropoietin has a specific activity equivalent to that of natural erythropoietin (78,000 units per mg protein), the biological assay corresponds to approximately 80 μg of erythropoietin protein per milliter.

In addition, supernatants from selected cell lines were assayed for immunologically reactive erythropoietin by competitive radioimmunoassay using a polyvalent anti-human-erythropoietin rabbit anti-serum (J.Cell. Physiol. 118:87-96, 1984). The amount of protein measured by the radioimmunoassay was equivalent to the protein level estimated by the biological assay. These data indicate that the transfected cell lines expressed and secreted erythropoietin protein that was greater than 98% active.

The recombinant erythropoietin produced by the transfected cells was further characterized to demonstrate that these cells were secreting authentic hormone. Selected cell lines were assayed for in vivo erythropoietin activity in exhypoxic polycythemic mice (Nature 191:1069-1087, 1961). Supernatants secreted from the cell lines had potent in vivo biological activity when assayed in the exhypoxic polycythemic mouse. In experiments using partially purified native erythropoietin, it had been noted previously that neuraminidase treatment completely abrogated erythropoietin activity when assayed in the intact animal (J.Biol.Chem. 247:5159-5160, 1958). The loss of activity presumably was due to increased clearance by the liver of the desialated hormone since neuraminidase-treated erythropoietin remained fully active in vitro. The observation of potent in vivo biological activity indicates that the transfected mammalian cell lines appropriately add carbohydrate and the terminal sialic acids to the erythropoietin protein during post-translational modification.

In separate experiments, the activity of erythropoietin in the in vitro biological assay was neutralized by a neutralizing anti-human erythropoietin antibody added to the culture.

The erythropoietin secreted into the supernatant of representative transfected cell lines was also assayed for proliferative effects on other marrow progenitor cells. Recombinant erythropoietin was assayed for its effect on a variety of progenitors from mouse and human marrow including erythroid colony-forming cells (CFU-E), erythroid burst-forming cells (BFU-E), granulocytemacrophage precursors (CFU-GM), and mixed-cell colony-forming cells (CFU-Mix) (J.Cell.Physiol.Suppl. 1:79-85, 1982; J.Cell.Physiol. 118:87-96, 1984). Erythroid stem cells exhibited a proliferation response to the recombinant erythropoietin that was parallel to the dose-response relationship found with natural erythropoietin. Neither CFU-GM nor CFU-Mix exhibited any proliferative response to the recombinant erythropoietin at concentrations up to 10 units per milliliter of assay cell culture.

When analyzed by SDS-PAGE under reducing or nonreducing conditions the purified recombinant erythropoietin electrophoresed identically to erythropoietin purified from urine of patients with aplastic anemia. Similar microheterogeneity of the proteins was observed, and the predominant species had identical molecular weights of 34 kD.

While the present invention has been described in conjunction with preferred embodiments, one of ordinary skill after reading the foregoing specification will be able to effect various changes, substitutions of equivalents, and other alterations to the compositions and methods set forth herein. It is therefore intended that the protection granted by Letters Patent hereon be limited only by the definition contained in the appended claims and equivalents thereof.

Claims

Claims for the following Contracting States : AT, BE, CH, DE, FR, GB, GR, IT, LI, LU, NL, SE

1. Substantially pure DNA or RNA consisting essentially of nucleotide sequence corresponding to the 2.4kb Apa I restriction fragment of a human erythropoietin gene.

2. DNA or RNA as claimed in Claim 1 wherein the Apa I restriction fragment consists essentially of the nucleotide sequence of either the sense strand shown in Figure 1 or the complementary RNA sequence thereof.

3. DNA or RNA as claimed in Claim 1 operably linked to a heterologous second nucleic acid sequence capable of effecting expression thereof.

4. DNA or RNA as claimed in Claim 3 wherein the second nucleic acid sequence is selected from one or more of the following: promoter sequences, enhancer sequences, polyadenylation sequences, selectable marker sequences, plasmids, viral and retroviral expression vectors, and retroviral trans-acting factors.

5. A cell line comprising cells containing the DNA or RNA of Claim 3 or Claim 4.

6. A cell line comprising cells stably transfected with the DNA or RNA of Claim 3 or Claim 4.

7. A cell line comprising cells of Claim 5 or Claim 6 selected from the group consisting of eukaryotic cells and bacteria.

8. A cell line of Claim 7 wherein the eukaryotic cells are of kidney origin.

9. A cell line of Claim 8 wherein the kidney cells are epithelial cells.

10. A method of expressing recombinant biologically active human erythropoietin comprising the steps of introducing the 2.4kb Apa I restriction fragment of a human erythropoietin gene into an expression vector, transfecting a host cell line with the expression vector containing the 2.4kb Apa I restriction fragment of a human erythropoietin gene, contacting transfected cells of the host cell line with culture medium to permit the cells to express erythropoietin, and recovering the expressed erythropoietin.

11. A method as claimed in Claim 10, wherein the cell line is capable of permitting a nominal yield of at least two million Units of erythropoietin per litre of incubating medium.

12. A method as claimed in Claim 10 or 11, wherein the Apa I restriction fragment is carried on a plasmid or a virus.

13. A method as claimed in Claim 10, 11 or 12 wherein the host cell line is selected from the group consisting of eukaryotic cells and bacteria.

14. A method as claimed in any one of Claims 10 to 13 wherein the transfected cells stably express erythropoietin.

15. A method as claimed in Claim 14 wherein the stable expression is achieved by challenging the cells to a high level of methotrexate.

16. A method as claimed in Claim 15 wherein the concentration of methotrexate used is in the order of 1mM.

Claims for the following Contracting State : ES

1. A process for the preparation of substantially pure DNA or RNA consisting essentially of nucleotide sequence corresponding to the 2.4kb Apa I restriction fragment of a human erythropoietin gene, the process comprising digesting a genomic clone of the human erythropoietin gene with Apa I.

2. A process as claimed in Claim 1 wherein the Apa I restriction fragment consists essentially of the nucleotide sequence of either the sense strand shown in Figure 1 or the complementary RNA sequence thereof.

3. A process as claimed in Claim 1 wherein the DNA or RNA is operably linked to a heterologous second nucleic acid sequence capable of effecting expression thereof.

4. A process as claimed in Claim 3 wherein the second nucleic acid sequence is selected from one or more of the following: promoter sequences, enhancer sequences, polyadenylation sequences, selectable marker sequences, plasmids, viral and retroviral expression vectors, and retroviral trans-acting factors.

5. A process for transfecting a cell line, the process comprising transfecting cells with DNA or RNA preparable by a process as claimed in Claim 3 or Claim 4.

6. A process as claimed in Claim 5, wherein the cell line is stably transfected.

7. A process as claimed in Claim 5 or Claim 6 wherein the cells are selected from the group consisting of eukaryotic cells and bacteria.

8. A process as claimed in Claim 7 wherein the eukaryotic cells are of kidney origin.

9. A process as claimed in Claim 8 wherein the kidney cells are epithelial cells.

10. A method of expressing recombinant biologically active human erythropoietin comprising the steps of introducing the 2.4kb Apa I restriction fragment of a human erythropoietin gene into an expression vector, transfecting a host cell line with the expression vector containing the 2.4kb Apa I restriction fragment of a human erythropoietin gene, contacting transfected cells of the host cell line with culture medium to permit the cells to express erythropoietin, and recovering the expressed erythropoietin.

11. A method as claimed in Claim 10, wherein the cell line is capable of permitting a nominal yield of at least two million Units of erythropoietin per litre of incubating medium.

12. A method as claimed in Claim 10 or 11, wherein the Apa I restriction fragment is carried on a plasmid or a virus.

13. A method as claimed in Claim 10, 11 or 12 wherein the host cell line is selected from the group consisting of eukaryotic cells and bacteria.

14. A method as claimed in any one of Claims 10 to 13 wherein the transfected cells stably express erythropoietin.

15. A method as claimed in Claim 14 wherein the stable expression is achieved by challenging the cells to a high level of methotrexate.

16. A method as claimed in Claim 15 wherein the concentration of methotrexate used is in the order of 1mM.

Revendications

Revendications pour les Etats contractants suivants : AT, BE, CH, DE, FR, GB, GR, IT, LI, LU, NL, SE

1. ADN ou ARN sensiblement pur consistant essentiellement en la séquence de nucléotides correspondant au fragment de restriction Apa I de 2,4 kb d'un gène d'érythropoïétine humaine.

2. ADN ou ARN suivant la revendication 1, dans lequel le fragment de restriction Apa I consiste essentiellement en la séquence de nucléotides soit du brin sens représenté à la Fig. 1, soit en la séquence d'ARN complémentaire de celui-ci.

3. ADN ou ARN suivant la revendication 1, lié de façon opérationnelle à une deuxième séquence hétérologue d'acides nucléiques capable d'opérer son expression.

4. ADN ou ARN suivant la revendication 3, dans lequel la deuxième séquence d'acides nucléiques est choisie parmi une ou plusieurs des suivantes : séquences promoteurs, séquences d'accélération, séquences de polyadénylation, séquences marqueurs sélectionnables, plasmides, vecteurs d'expression viraux et rétroviraux et facteurs d'action trans rétroviraux.

5. Lignée de cellules comprenant des cellules contenant l'ADN ou ARN suivant la revendication 3 ou 4.

6. Lignée de cellules comprenant des cellules transfectées de façon stable par L'ADN ou ARN suivant la revendication 3 ou 4.

7. Lignée de cellules comprenant des cellules suivant la revendication 5 ou 6, sélectionnées dans la classe formée par les cellules eucaryotes et les bactéries.

8. Lignée de cellules suivant la revendication 7, dans laquelle les cellules eucaryotes sont d'origine rénale.

9. Lignée de cellules suivant la revendication 8, dans laquelle les cellules rénales sont des cellules épithéliales.

10. Procédé d'expression de l'érythropoïétine humaine biologiquement active recombinante, comprenant les stades d'introduction du fragment de restriction Apa I de 2,4 kb d'un gène d'érythropoïétine humaine dans un vecteur d'expression, de transfection d'une lignée de cellules hôtes à l'aide du vecteur d'expression contenant le fragment de restriction Apa I de 2,4 kb d'un gène d'érythropoïétine humaine, de mise en contact de cellules transfectées de la lignée de cellules hôtes avec un milieu de culture pour permettre aux cellules d'exprimer l'érythropoïétine et de collecte de l'érythropoïétine exprimée.

11. Procédé suivant la revendication 10, dans lequel la lignée de cellules est capable de permettre une production nominale d'au moins deux millions d'unités d'érythropoïétine par litre de milieu d'incubation.

12. Procédé suivant la revendication 10 ou 11, dans lequel le fragment de restriction Apa I est porté sur un plasmide ou un virus.

13. Procédé suivant la revendication 10, 11 ou 12, dans lequel la lignée de cellules hôtes est choisie dans la classe formée par les cellules eucaryotes et les bactéries.

14. Procédé suivant l'une quelconque des revendications 10 à 13, dans lequel les cellules transfectées de façon stable expriment l'érythropoïétine.

15. Procédé suivant la revendication 14, dans lequel l'expression stable est obtenue en exposant les cellules à une concentration élevée de méthotrexate.

16. Procédé suivant la revendication 15, dans lequel la concentration de méthotrexate utilisée est de l'ordre de 1 mM.

Revendications pour l'Etat contractant suivant : ES

1. Procédé de préparation d'un ADN ou ARN sensiblement pur consistant essentiellement en la séquence de nucléotides correspondant au fragment de restriction Apa I de 2,4 kb d'un gène d'érythropoïétine humaine, lequel procédé comprend la digestion d'un clone génomique du gène d'érythropoïétine humaine avec Apa I.

2. Procédé suivant la revendication 1, dans lequel le fragment de restriction Apa I consiste essentiellement en la séquence de nucléotides soit du brin sens représenté à la Fig. 1, soit en la séquence d'ARN complémentaire de celui-ci.

3. Procédé suivant la revendication 1, dans lequel l'ADN ou ARN est lié de façon opérationnelle à une deuxième séquence hétérologue d'acides nucléiques capable d'opérer son expression.

4. Procédé suivant la revendication 3, dans lequel la deuxième séquence d'acides nucléiques est choisie parmi une ou plusieurs des suivantes : séquences promoteurs, séquences d'accélération, séquences de polyadénylation, séquences marqueurs sélectionnables, plasmides, vecteurs d'expression viraux et rétroviraux et facteurs d'action trans rétroviraux.

5. Procédé pour transfecter une lignée de cellules, lequel procédé comprend la transfection des cellules avec de l'ADN ou ARN qui peut être préparé par un procédé suivant la revendication 3 ou 4.

6. Procédé suivant la revendication 5, dans lequel la lignée de cellules est transfectée de façon stable.

7. Procédé suivant la revendication 5 ou 6, dans lequel les cellules sont sélectionnées dans la classe formée par les cellules eucaryotes et les bactéries.

8. Procédé suivant la revendication 7, dans lequel les cellules eucaryotes sont d'origine rénale.

9. Procédé suivant la revendication 8, dans lequel les cellules rénales sont des cellules épithéliales.

10. Procédé d'expression de l'érythropoïétine humaine biologiquement active recombinante, comprenant les stades d'introduction du fragment de restriction Apa I de 2,4 kb d'un gène d'érythropoïétine humaine dans un vecteur d'expression, de transfection d'une lignée de cellules hôtes à l'aide du vecteur d'expression contenant le fragment de restriction Apa I de 2,4 kb d'un gène d'érythropoïétine humaine, de mise en contact de cellules transfectées de la lignée de cellules hôtes avec un milieu de culture pour permettre aux cellules d'exprimer l'érythropoïétine et de collecte de l'érythropoïétine exprimée.

11. Procédé suivant la revendication 10, dans lequel la lignée de cellules est capable de permettre une production nominale d'au moins deux millions d'unités d'érythropoïétine par litre de milieu d'incubation.

12. Procédé suivant la revendication 10 ou 11, dans lequel le fragment de restriction Apa I est porté sur un plasmide ou un virus.

13. Procédé suivant la revendication 10, 11 ou 12, dans lequel la lignée de cellules hôtes est choisie dans la classe formée par les cellules eucaryotes et les bactéries.

14. Procédé suivant l'une quelconque des revendications 10 à 13, dans lequel les cellules transfectées de façon stable expriment l'érythropoïétine.

15. Procédé suivant la revendication 14, dans lequel l'expression stable est obtenue en exposant les cellules à une concentration élevée de méthotrexate.

16. Procédé suivant la revendication 15, dans lequel la concentration de méthotrexate utilisée est de l'ordre de 1 Mm.

Patentansprüche

Patentansprüche für folgende Vertragsstaaten : AT, BE, CH, DE, FR, GB, GR, IT, LI, LU, NL, SE

1. Im wesentlichen reine DNA oder RNA, hauptsächlich bestehend aus einer Nucleotidsequenz, welche dem 2.4kb Apa I Restriktionsfragment eines menschlichen Erythropoietin-Gens entspricht.

2. DNA oder RNA nach Anspruch 1, wobei das Apa I Restriktionsfragment hauptsächlich aus der Nucleotidsequenz entweder des in Figur 1 dargestellten codierenden Strangs oder der dazu komplementären RNA-Sequenz besteht.

3. DNA oder RNA nach Anspruch 1, die funktionell mit einer heterologen sekundären Nucleinsäuresequenz verbunden ist, welche deren Expression bewirken kann.

4. DNA oder RNA nach Anspruch 3, wobei die sekundäre Nucleinsäuresequenz aus einer oder mehreren der folgenden Gruppen gewählt wird: Promotorsequenzen, Verstärkersequenzen, Polyadenylierungssequenzen, selektierbaren Markersequenzen, Plasmiden, viralen und retroviralen Expressionsvektoren und retroviralen Transaktionsfaktoren.

5. Zellinie, die Zellen umfaßt, welche die DNA oder RNA nach Anspruch 3 oder Anspruch 4 enthalten.

6. Zellinie, die Zellen umfaßt, welche mit der DNA oder RNA nach Anspruch 3 oder Anspruch 4 stabil transfektiert sind.

7. Zellinie, die Zellen nach Anspruch 5 oder Anspruch 6 umfaßt, welche aus der Gruppe, bestehend aus eukaryotischen Zellen und Bakterien, gewählt werden.

8. Zellinie nach Anspruch 7, wobei die eukaryotischen Zellen von der Niere stammen.

9. Zellinie nach Anspruch 8, wobei die Nierenzellen Epithelzellen sind.

10. Verfahren zur Expression von rekombinantem, biologisch aktiven, menschlichen Erythropoietin, das folgende Schritte umfaßt:

    Einführen des 2.4kb Apa I Restriktionsfragments eines menschlichen Erythropoietin-Gens in einen Expressionsvektor;

    Transfektion einer Wirtszellinie mit dem Expressionsvektor, der das 2.4kb Apa I Restriktionsfragment eines menschlichen Erythropoietin-Gens enthält;

    Kontaktieren der transfektierten Zellen der Wirtszellinie mit Kulturmedium, so daß die Zellen Erythropoietin exprimieren können; und

    Gewinnung des exprimierten Erythropoietins.

11. Verfahren nach Anspruch 10, wobei die Zellinie eine nominale Ausbeute von zumindest zwei Millionen Einheiten von Erythropoietin pro Liter Inkubationsmedium ermöglichen kann.

12. Verfahren nach Anspruch 10 oder 11, wobei das Apa I Restriktionsfragment von einem Plasmid oder einem Virus getragen wird.

13. Verfahren nach Anspruch 10, 11 oder 12, wobei die Wirtszellinie aus der Gruppe, bestehend aus eukaryotischen Zellen und Bakterien, gewählt wird.

14. Verfahren nach einem der Ansprüche 10 bis 13, wobei die transfektierten Zellen stabil Erythropoietin exprimieren.

15. Verfahren nach Anspruch 14, wobei die stabile Expression erzielt wird, indem die Zellen einem hohen Spiegel von Methotrexat ausgesetzt werden.

16. Verfahren nach Anspruch 15, wobei die verwendete Methotrexatkonzentration im Bereich von 1mM liegt.

Patentansprüche für folgenden Vertragsstaat : ES

1. Verfahren zur Herstellung von im wesentlichen reiner DNA oder RNA, hauptsächlich bestehend aus einer Nucleotidsequenz, welche dem 2.4kb Apa I Restriktionsfragment eines menschlichen Erythropoietin-Gens entspricht, wobei das Verfahren das Digerieren eines genomischen Klons des menschlichen Erythropoietin-Gens mit Apa I umfaßt.

2. Verfahren nach Anspruch 1, wobei das Apa I Restriktionsfragment hauptsächlich aus der Nucleotidsequenz entweder des in Figur 1 dargestellten codierenden Strangs oder der dazu komplementären RNA-Sequenz besteht.

3. Verfahren nach Anspruch 1, wobei die DNA oder RNA funktionell mit einer heterologen sekundären Nucleinsäuresequenz verbunden ist, welche deren Expression bewirken kann.

4. Verfahren nach Anspruch 3, wobei die sekundäre Nucleinsäuresequenz aus einer oder mehreren der folgenden Gruppe gewählt wird: Promotorsequenzen, Verstärkersequenzen, Polyadenylierungssequenzen, selektierbaren Markersequenzen, Plasmiden, viralen und retroviralen Expressionsvektoren und retroviralen Transaktionsfaktoren.

5. Verfahren zur Transfektion einer Zellinie, wobei das Verfahren das Transfektieren von Zellen mit DNA oder RNA umfaßt, die durch ein Verfahren nach Anspruch 3 oder Anspruch 4 hergestellt werden kann.

6. Verfahren nach Anspruch 5, wobei die Zellinie stabil transfektiert ist.

7. Verfahren nach Anspruch 5 oder Anspruch 6, wobei die Zellen aus der Gruppe, bestehend aus eukaryotischen Zellen und Bakterien, gewählt werden.

8. Verfahren nach Anspruch 7, wobei die eukaryotischen Zellen von der Niere stammen.

9. Verfahren nach Anspruch 8, wobei die Nierenzellen Epithelzellen sind.

10. Verfahren zur Expression von rekombinantem, biologisch aktiven, menschlichen Erythropoietin, das folgende Schritte umfaßt:

    Einführen des 2.4kb Apa I Restriktionsfragments eines menschlichen Erythropoietin-Gens in einen Expressionsvektor;

    Transfektion einer Wirtszellinie mit dem Expressionsvektor, der das 2.4kb Apa I Restriktionsfragment eines menschlichen Erythropoietin-Gens enthält;

    Kontaktieren der transfektierten Zellen der Wirtszellinie mit Kulturmedium, so daß die Zellen Erythropoietin exprimieren können; und

    Gewinnung des exprimierten Erythropoietins.

11. Verfahren nach Anspruch 10, wobei die Zellinie eine nominale Ausbeute von zumindest zwei Millionen Einheiten von Erythropoietin pro Liter Inkubationsmedium ermöglichen kann.

12. Verfahren nach Anspruch 10 oder 11, wobei das Apa I Restriktionsfragment von einem Plasmid oder einem Virus getragen wird.

13. Verfahren nach Anspruch 10, 11 oder 12, wobei die Wirtszellinie aus der Gruppe, bestehend aus eukaryotischen Zellen und Bakterien, gewählt wird.

14. Verfahren nach einem der Ansprüche 10 bis 13, wobei die transfektierten Zellen stabil Erythropoietin exprimieren.

15. Verfahren nach Anspruch 14, wobei die stabile Expression erzielt wird, indem die Zellen einem hohen Spiegel von Methotrexat ausgesetzt werden.

16. Verfahren nach Anspruch 15, wobei die verwendete Methotrexatkonzentration im Bereich von 1mM liegt.

DNA:epo:apa.gene
DNA LENGTH: 2426

FIRST NUCLEOTIDE: +1

First at.	10	20	30	40	50	60	70	80	90	100
+1	CCCGGTGTGG	TCACCCGGCG	CGCCCCAGGT	CGCTGAGGGA	CCCCGGCCAG	GCGCGGAGAT	GGGGGTGCAC	GGTGAGTACT	CGCGGGCTGG	GCGCTCCCGC
+101	CCGCCCGGGT	CCCTGTTTGA	GCGGGGATTT	AGCGCCCCGG	CTATTGGCCA	GGAGGTGGCT	GGGTTCAAGG	ACCGGCGACT	TGTCAAGGAC	CCCGGAAGGG
+201	GGAGGGGGGT	GGGGCAGCCT	CCACGTGCCA	GCGGGGACTT	GGGGGAGTCC	TTGGGGATGG	CAAAAACCTG	ACCTGTGAAG	GGGXCACAGT	TTGGGGGTTG
+301	AGGGGAAGAA	GGTTTGGGGG	GTTCTGCTGT	GCCAGTGGAG	AGGAAGCTGA	TAAGCTGATA	ACCTGGGCGC	TGGAGCCACC	ACTTATCTGC	CAGAGGGGAA
+401	GCCTCTGTCA	CACCAGGATT	GAAGTTTGGC	CGGAGAAGTG	GATGCTGGTA	GCCTGGGGGT	GGGGTGTGCA	CACGGCAGCA	GGATTGAATG	AAGGCCAGGG
+501	AGGCAGCACC	TGAGTGCTTG	CATGGTTGGG	GACAGGAAGG	ACGAGCTGGG	GCAGAGACGT	GGGGATGAAG	GAAGCTGTCC	TTCCACAGCC	ACCCTTCTCC
+601	CTCCCCGCCT	GACTCTCAGC	CTGGCTATCT	GTTCTAGAAT	GTCCTGCCTG	GCTGTGGCTT	CTCCTGTCCC	TGCTGTCGCT	CCCTCTGGGC	CTCCCAGTCC
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+801	GCACATTCCA	CAGAACTCAC	GCTCAGGGCT	TCAGGGAACT	CCTCCCAGAT	CCAGGAACCT	GGCACTTGGT	TTGGGGTGGA	GTTGGGAAGC	TAGACACTGC
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+1001	TTCAGGGACC	CTTGACTCCC	CGGGCTGTGT	GCATTTCAGA	CGGGCTGTGC	TGAACACTGC	AGCTTGAATG	AGAATATCAC	TGTCCCAGAC	ACCAAAGTTA
+1101	ATTTCTATGC	CTGGAAGAGG	ATGGAGGTGA	GTTCCTTTTT	TTTTTTTTTT	CCTTTCTTTT	GGAGAATCTC	ATTTGCGAGC	CTGATTTTGG	ATGAAAGGGA

Fig. 1A.

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+1501   CTCAGTGACA GAGTGAGGCC CTGTCTCAAA AAAGAAAAGA AAAAAGAAAA ATAATGAGGG CTGTATGGAA TACGTTCATT ATTCATTCAC TCACTCACTC
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+1701   CATCCCTCAG CTGACTCCCA GAGTCCACTC CCTGTAGGTC GGGCAGCAGG CCGTAGAAGT CTGGCAGGGC CTGGCCCTGC TGTCGGAAGC TGTCCTGCGG
+1801   GGCCAGGCCC TGTTGGTGAA CTCTTCCCAG CCGTGGGAGC CCCTGCAGCT GCATGTGGAT AAAGCCGTCA GTGGCCTTCG CAGCCTCACC ACTCTGCTTC
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+2001   TTCCTTCCCT TTCTGTGGCA CTGCAGCGAC CTCCTGTTTC CTCCTTGGCA GAAGGAAGCC ATCTCCCCTC CAGATGCGGC CTCAGCTGCT CCACTCCGAA
+2101   CAATCACTGC TGACACTTTC CGCAAACTCT TCCGAGTCTA CTCCAATTTC CTCCGGGGAA AGCTGAAGCT GTACACAGGG GAGGCCTGCA GGACAGGGGA
+2201   CAGATGACCA GGTGTGTCCA CCTGGGCATA TCCACCACCT CCCTCACCAA CATTGCTTGT GCCACACCCT CCCCCGCCAC TCCTGAACCC CGTCGAGGGG
+2301   CTCTCAGCTC AGCGCCAGCC TGTCCCATGG ACACTCCAGT GCCACCAATG ACATCTCAGG GGCCAGAGGA ACTGTCCAGA GAGCAACTCT GAGATCTAAG
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Fig. 1B.

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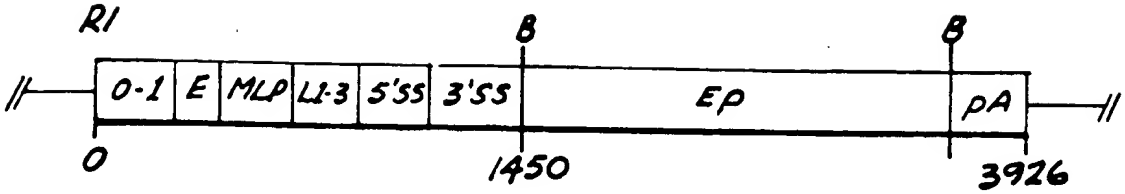
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3'SS
EP
pA
0
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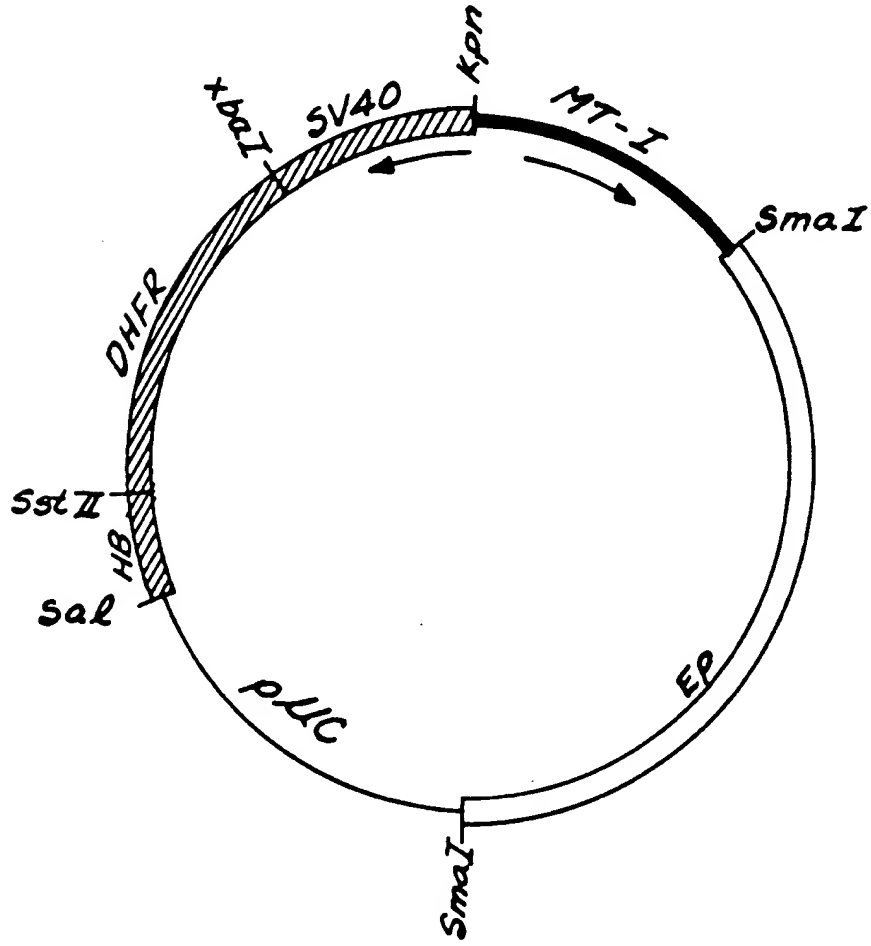
3926

fig. 2.


Kpn
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MT-I
XbaI
SmaI
DHFR
SstII
HB
Sal
pUC
EP
SmaI

fig. 3.